Nrf2 regulates the sensitivity of death receptor signals by affecting intracellular glutathione levels

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Nrf2 regulates the sensitivity of death receptor signals by affecting intra-cellular glutathione levels.

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Abstract

Nrf2 is a basic leucine zipper transcriptional activator that is essential for the coordinate transcriptional induction of various antioxidant drug metabolizing enzymes. Numerous studies have firmly established Nrf2's importance in the protection from oxidative stress and certain chemical insults. Given the protective function of Nrf2, surprisingly few studies have focused on the relationship between Nrf2 and apoptosis. Therefore, we analyzed how Nrf2 influences Fas signaling using Nrf2 deficient T cells. At a concentration of 1 μg/ml, anti-Fas antibody induced 60% of cell death in Nrf2 deficient cultured thymocytes while, using the same treatment, only 40% of Nrf2 wild type thymocytes died (P <0.05). Nrf2 deficiency enhances the sensitivity of Fas-mediated apoptosis in T cells. Next we examined the effect of Nrf2 deficiency during hepatocellular apoptosis *in vivo*. In comparison to wild-type mice, Nrf2 deficient mice displayed more severe hepatitis after induction with anti-Fas antibody or TNF-α. The enhanced sensitivity to anti-Fas or TNF-α stimulation was restored by pre-administration of glutathione ethyl monoester, a compound capable of passing the cell membrane and up-regulating the intra-cellular levels of glutathione. The results indicated that Nrf2 activity regulates the sensitivity of death signals by means of intracellular glutathione

levels.

Nrf2 (NF-E2 related factor 2) was originally defined as a basic leucine zipper (b-Zip) transcriptional activator which recognizes the NF-E2 (nuclear factor erythroid 2) binding site (Moi *et al*., 1994; Itoh *et al*., 1995). Nrf2 is essential for the coordinate transcriptional induction of various antioxidant and phase II drug metabolizing enzymes through the antioxidant response element/electrophile response element (ARE/EpRE) (Itoh *et al*., 1997; Itoh *et al*., 1999). ARE/EpRE sequences have been characterized within the proximal regulatory sequences of genes encoding the antioxidant enzymes glutathione S-transferase (GST) (Rushmore *et al*., 1990), NAD(P)H quinone oxidoreductase (NQO1) (Favreau *et al*., 1991), heme oxygenase-1 (HO-1) (Prestera *et al*., 1995), γ-glutamylcysteine synthetase (Chan and Kwong, 2000) and cystine membrane transporter (system X_c^-) (Ishii *et al*., 2000). The ARE also regulates a wide range of metabolic responses to oxidative stress caused by reactive oxygen species (ROS) or electrophiles (Ishii *et al*., 2000). As a consequence of inadequate induction of these molecules, Nrf2 deficient mice are sensitive to high oxidative stress and drug-induced stress (Ishii *et al*., 2000; Cho *et al*., 2002; Enomoto *et al*., 2001).

Glutathione (L-γ-glutamyl-cysteinyl-glycine, GSH) is a tripeptide, intracellular, non-protein thiol that has a central role in sulfhydryl homeostasis. GSH serves as the major cytosolic antioxidant and defends against xenobiotics

by acting as a substrate during phase II conjugation reactions (Jones *et al*., 1986; Meister, 1992). Numerous cellular functions are modulated by the glutathione disulfide system including, regulation of enzymes vital to metabolism, cell growth, gene transcription, and apoptosis (Droge *et al*., 1994; Uhlig and Wendel 1992; Hall, 1999). Therefore, cells tightly regulate the synthesis, utilization, and export of GSH. The intracellular concentration of GSH is maintained within the millimolar range under normal conditions (Anderson, 1997). Synthesis of GSH is achieved by the consecutive action of the ATP-dependent enzymes, γ–glutamylcysteine synthetase and glutathione synthetase. It was reported that levels of both these key enzymes are affected by the activity of Nrf2 (Chan and Kwong, 2000). In addition, the expression of system X_c^- is controlled by Nrf2 (Ishii *et al*., 2000). System X_c^- regulates the concentration of intracellular cystine, a component substrate for GSH synthesis. Based upon these findings, Nrf2 is thought to be one of the main regulators of intracellular GSH levels.

The cytokine receptor Fas (APO-1/CD95) belongs to the tumor necrosis factor (TNF)/nerve growth factor receptor superfamily. Certain members of this family, including Fas, are referred to as death receptors since they share a cytoplasmic death domain involved in apoptotic signaling (Schulze-Osthoff *et al*., 1998). Fas is expressed by lymphoid and nonlymphoid cells and is regarded as a principal trigger for apoptotic cell death of lymphoid cells and hepatocytes

(Galle *et al*., 1995; Nagata, 1997). In both human and murine liver, Fas and TNF-receptor I (TNF-RI) can independently trigger apoptosis. Indeed, the administration of an activating anti-Fas antibody to mice leads to lethal liver destruction within hours due to extensive apoptosis of hepatocytes (Ogasawara *et al*., 1993; Leist *et al*., 1996). Upon ligand binding and trimerization of Fas, the intracellular death domain of the receptor associates with several proteins forming a death-inducing signaling complex (DISC) through which the recruitment and activation of caspase-8 is initiated (Medema *et al*., 1997; Schulze-Osthoff *et al*., 1998). This event, in turn, triggers a cascade of proteolytic interconversions of procaspases, including downstream caspases such as caspase-3 and –7, which finally execute the proteolytic cleavage of various structural and signal proteins (Zhivotovsky *et al*., 1997; Thronberry and Lazebnik, 1998; Hirata *et al*., 1998; Widmann *et al*.,1998). The ultimate outcome of this cascade is apoptosis. TNF-α also plays an integral role in the injury and cell death that occurs in hepatocytes following toxin-induced liver damage. TNF-α induced cell death is mainly transduced through TNF-RI and the signaling pathway is similar to Fas induced apoptosis (Schulze-Osthoff *et al*., 1998).

There have been several reports describing the modulation of Fas- or TNF-RI-mediated apoptosis by GSH. According to these reports, acute GSH

depletion induced insensitivity to death receptor stimulation (Hentze *et al*., 1999; Hentze *et al*., 2000), while, during prolonged GSH depletion increased death receptor mediated apoptosis was observed (Chiba *et al*., 1996; Colell *et al*., 1998; Xu *et al*., 1998). The reports strongly indicated that Nrf2 activity may influence the sensitivity of death signaling, but the relationship between Nrf2 and apoptotic signaling was not clearly characterized. Recently, Kotlo *et al.* demonstrated that inactivation of Nrf2 by antisense or by using a membrane permeable dominant-negative polypeptide sensitized cells while conversely, overexpression of Nrf2 protected cells from Fas-induced apoptosis. In addition, they demonstrated that N-acetyl L-cysteine (NAC), a precursor to GSH, protected cells from Fas-mediated killing in HeLa cells (Kotlo *et al*., 2003).

In this report, we have analyzed the sensitivity of death signaling (Fas and TNF-α-mediated apoptosis) in Nrf2 deficient mice and demonstrated a high susceptibility to death signals *in vivo*. Pre-administration of glutathione ethyl monoester (GSH-OEt), which up-regulates GSH, canceled the observed enhanced sensitivity. This indicates that the low intracellular GSH levels which resulted from Nrf2 deficiency is a primary cause for the susceptibility.

To investigate the role of Nrf2 in Fas-mediated apoptosis, cultured Nrf2 deficient thymocytes were examined in the presence of an agonistic anti-Fas antibody. Our results showed that a deficiency in Nrf2 increased the percentage

of apoptotic thymocytes following Fas stimulation (Figure 1A). Anti-Fas antibody, at a concentration of 1 μg/ml, induced 60% of cell death in thymocytes from Nrf2 deficient mice while, using the same treatment, 40% of cells from wild type mice died (P <0.05). Recently, it has been found that prolonged GSH depletion enhances Fas-mediated apoptosis (Haouzi *et al*., 2001). We and others reported that intracellular GSH levels were reduced in Nrf2 deficient cells (Chan and Kwong, 2000; Ishii *et al*., 2000). Therefore, we suspected that the observed sensitivity of cultured thymocytes to Fas-mediated apoptosis could be due to a depletion of cellular GSH levels resulting from a deficiency in Nrf2. Administration of GSH-OEt can increase the intracellular GSH concentration *in vitro* and *in vivo* (Rahman *et al*., 2001; Chen *et al*., 2001). Therefore, we examined the effect of GSH-OEt treatment on Fas-mediated apoptosis in Nrf2 deficient thymocytes. The addition of GSH-OEt to Nrf2 deficient thymocytes re-established the sensitivity of Fas-mediated apoptosis to the level of wild type mice thymocytes (Figure 1B). These results suggest that the enhanced sensitivity of the Fas signal in Nrf2 deficient thymocytes was due to a decrease in the intracellular GSH levels. However, due to the complicated nature of the GSH system, it is not possible to exclude the possibility that the administered GSH affected the adequate induction of some alternative adaptor molecule(s), whose function could be to prevent Fas-mediate apoptosis. To

confirm the hypothesis that the enhanced sensitivity of the Fas signal was due to a decrease in the intracellular GSH levels, we analyzed mice chronically treated with buthionine sulfoximine (BSO). BSO is a specific GSH synthesis inhibitor that when administered to mice serves as a model of chronic GSH depletion (Sanjay *et al*., 2000). Wild type mice were given water supplemented with BSO (400 mg/kg/day) for 6 weeks. After 6weeks, thymocytes were isolated from BSO treated and control (untreated) wild type mice. Analyzes showed that Fas stimulation resulted in a larger percentage of apoptotic cells among BSO treated thymocytes compared to wild type control (Figure 1C). Furthermore, the addition of GSH-OEt to BSO treated thymocytes re-established the sensitivity of Fas-mediated apoptosis to the level of BSO untreated mice thymocytes (Figure 1D). These results support the hypothesis that chronic GSH depletion enhances Fas-mediated apoptosis *in vitro.*

In the same experiment we also determined the intracellular levels of GSH of the thymocytes (Figure 1E). The GSH level of Nrf2 deficient thymocytes was decreased to almost the same level as that of BSO treated wild type thymocytes. GSH-OEt addition increased the GSH levels of Nrf2 deficient and BSO treated thymocytes to the same level as that of Nrf2 wild type thymocytes. These results also support the hypothesis that chronic GSH depletion enhanced Fas-mediated apoptosis *in vitro*.

It is well known that the administration of anti-Fas antibody to mice can induce fulminant hepatitis as a result of Fas-mediated apoptosis (Ogasawara *et al*., 1993). The effect of hepatocellular apoptosis *in vivo* triggered by anti-Fas antibody in Nrf2 deficient mice was examined. Anti-Fas antibody (Jo-2, 2μg/mouse) was administered intraperitoneally to Nrf2 deficient and wild type mice. Serum alanine aminotransferase (ALT) activities were determined as indices of hepatotoxicity. Serum ALT activity after Fas stimulation was significantly increased in Nrf2 deficient mice compared to Nrf2 wild type mice ($P<0.05$) (Table 1). At 12 hours after anti-Fas antibody administration, ALT activity in sera from Nrf2 deficient mice had increased to over 10000 IU/l, while those of wild type mice was around 5000 IU/l. Three out of five Nrf2 deficient mice were already dead at eight hour after injection. To prove the enhancement of hepatitis in Nrf2 deficient mice was also due to a decrease in intracellular GSH levels, GSH-OEt was administrated before anti-Fas antibody injection. The addition of GSH-OEt blocked the enhancement of Fas induced fulminant hepatitis suggesting that a low level of intracellular GSH might be the cause of the enhancement. To confirm this theory, we investigated the effect of chronically treating the mice with BSO. Serum ALT activity after Fas stimulation was significantly increased in BSO treated wild type mice compared to untreated wild type mice ($P<0.05$) (Table 1). The addition of

GSH-OEt cancelled the enhancement of Fas induced fulminant hepatitis in BSO treated mice. These results suggest that a low level of intracellular GSH could be the cause of the enhancement *in vivo*.

Fas and TNF-RI share homology at their cytoplasmic death domain, a region necessary for apoptotic signaling (Schulze-Osthoff *et al.*, 1998). D-galactosamine (GalN) is well known to increase the susceptibility of mice to hepatotoxicity and the lethal effects of TNF-α. Therefore, the effect of TNF-α on hepatocellular apoptosis in GalN sensitized Nrf2 deficient mice was examined. TNF-α (0.5μg/mice) was administered intraperitoneally to GalN sensitized Nrf2 deficient and wild type mice. The result showed that serum ALT activity after TNF-α administration was significantly increased in Nrf2 deficient mice compared to wild type mice. The mean ALT activity in the sera from Nrf2 deficient mice was 8732 ± 4413 IU/l at 12 hour after induction (Table 2). On the other hand, the ALT activity of wild type mice was less than 2000 IU/l when taken at the same time point. This represents a four-fold difference of ALT activity between these two groups. GSH-OEt addition decreased the ALT activity in Nrf2 deficient mice to almost the same level as that of wild type mice. To investigate the influence of GSH depletion, we examined wild type mice chronically treated with BSO. Serum ALT activity after TNF-α stimulation was significantly increased in BSO treated wild type mice compared to untreated

wild type mice (P<0.05) (Table 2). The addition of GSH-OEt decreased the enhancement of TNF-α induced fulminant hepatitis in BSO treated wild type mice. This result suggested that a low level of intracellular GSH would be the cause of the enhanced TNF-α–mediated apoptosis.

Histopathological examination revealed that the number of apoptotic hepatocytes was increased in Nrf2 deficient mice compared to wild type mice (Figure 2). Massive hepatocyte apoptosis, as judged by the frequent appearance of nuclear fragmentation and a hyperchromatic nuclear membrane was observed in Nrf2 deficient mice (Figure 2B). Further major pathological signs included diffuse congestive hemorrhagic foci (Figure 2B). In contrast, liver specimens from a comparable area taken from mice injected with GSH-OEt before TNF-α administration showed a morphology (Figure 2C) that was indiscernible from that of wild type mice (Figure 2A). BSO treated wild type mice revealed massive hepatocyte apoptosis by TNF-α administration as well as Nrf2 deficient mice (Figure 2D). The administration of GSH-OEt to BSO treated wild type mice re-established the sensitivity of TNF-α-mediated apoptosis to the level of wild type mice as judged by histological appearance (data not shown). The histological analyses also support that Nrf2 deficiency enhances the sensitivity of TNF-α-mediated apoptosis as a consequence of GSH depletion.

To confirm the effect of GSH on TNF-α mediated apoptosis *in vivo*, we determined the total liver GSH levels (Figure 2E). Mice were sacrificed 4 hours after TNF-α and GalN administration. Livers were homogenized and total GSH levels were measured. The GSH level of Nrf2 deficient livers was decreased to almost the same level as that of BSO treated wild type livers. GSH-OEt addition increased the GSH levels of Nrf2 deficient and BSO treated wild type livers to almost the same level as that of Nrf2 wild type livers. These results suggest that chronic GSH depletion can enhance TNF-α-mediated apoptosis *in vivo*.

An accumulating number of reports describe the relationship between Nrf2 and various kinds of stresses. Cho and colleagues reported that Nrf2 had a key protective role during hyperoxic lung injury (Cho *et al*., 2002). Enomoto et al. and Chan et al. clearly demonstrated an enhanced susceptibility to acetaminophen in Nrf2 deficient mice (Enomoto *et al*., 2001; Chan *et al*., 2001). We also reported that Nrf2 female mice developed autoimmune glomerulonephritis probably due to a susceptibility to oxidative stress (Yoh *et al*., 2001). In addition, Ramos-Gomez et al. showed that Nrf2 was central to the constitutive and inducible expression of phase two enzymes *in vivo* and dramatically influences susceptibility to carcinogens (Ramos-Gomez *et al*., 2001). However few studies has been reported the relationship between Nrf2 and apoptosis. This study is the first report describing Nrf2 regulates the sensitivity

of death receptor signals through intra-cellular glutathione levels *in vivo*. The inhibiton of death signal is a novel role of Nrf2. Possibly, the observed enhancement in death signals caused by a deficiency in Nrf2 is a safety mechanism to eliminate cells which have accumulated damage as a consequence of an inadequate stress response. Further analyses must be performed to explore the relationship between apoptotic regulation by Nrf2 and various stresses including diseases.

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Table 1

Hepatotoxicity and lethality in Nrf2 deficient mice induced by anti-Fas antibody

Treatment		Time (h)	ALT (IU/l) Nrf2 deficient	ALT (IU/l) wild type	ALT (IU/l) BSO treated wild type	Survival rate (survived/all) Nrf2 deficient	Survival rate (survived/all) wild type	Survival rate (survived/all) BSO treated wild type
GSH-OEt(-)	anti-Fas	0	42±9	35±8	37±4	5/5	5/5	5/5
GSH-OEt(-)	anti-Fas	4	1301±329*	626±100	1273±240 *	5/5	5/5	5/5
GSH-OEt(-)	anti-Fas	8	8110±2101*	3560±1175	8642±2346*	3/5	5/5	5/5
GSH-OEt(-)	anti-Fas	12	12566±4848*	4582±835	9739±4342*	3/5	4/5	4/5
GSH-OEt(+)	anti-Fas	0	26±10	32±8	25±9	5/5	5/5	5/5
GSH-OEt(+)	anti-Fas	4	671±106	431±96	398±119	5/5	5/5	5/5
GSH-OEt(+)	anti-Fas	8	2621±766	1832±805	2798±468	5/5	5/5	5/5
GSH-OEt(+)	anti-Fas	12	4944±563	3456±934	4878±889	5/5	5/5	5/5

Anti-Fas antibody (Jo-2, 2μg/mouse) was administered intraperitoneally as a mixture in 0.2 ml of pyrogen-free saline to 12-week-old Nrf2 deficient and wild type (BSO treated and untreated) mice. At 0, 4, 8 and 12 h after injection, blood was collected and serum ALT activities were determined as indices of hepatotoxicity using an automated analyzer (DRY-CHEM 3500; Fuji Film). GSH-OEt (20mg/mouse) was injected intravenously 2 h before anti-Fas antibody injection to increase the intracellular GSH level. Results are expressed

as mean ± SD for 3 to 5 mice. Survival rate indicates number of survived/all mice. (*) Significantly different from wild type (BSO untreated) mice (P<0.05).

Table 2

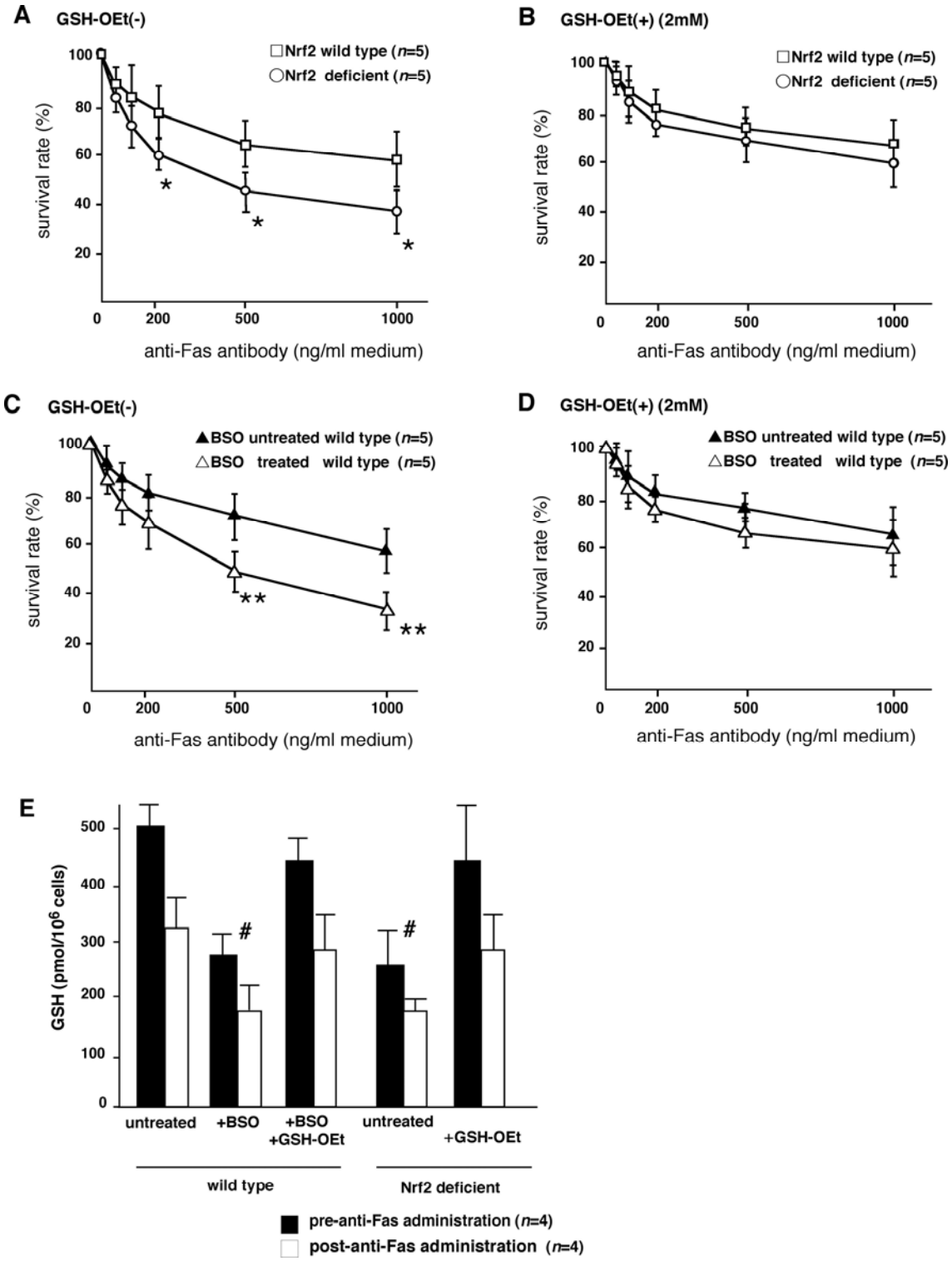
Hepatotoxicity and lethal effect in Nrf2 deficient and BSO treated mice induced by TNF- α

			ALT (IU/l)			Survival rate (survived/all)		
Treatment		Time (h)	Nrf2 deficient	wild type	BSO treated wild type	Nrf2 deficient	wild type	BSO treated wild type
GSH-OEt(-)	TNF-α	0	31±8	41±8	42±4	5/5	5/5	5/5
GSH-OEt(-)	TNF-α	4	942±301 *	235±104	873±276 *	5/5	5/5	5/5
GSH-OEt(-)	TNF-α	8	8207±1431*	1642±815	5642±2142*	4/5	5/5	4/5
GSH-OEt(-)	TNF-α	12	8732±4413*	1901±1403	6739±3732*	3/5	4/5	4/5
GSH-OEt(+)	TNF-α	0	25±9	37±11	33±7	5/5	5/5	5/5
GSH-OEt(+)	TNF-α	4	363±182	212±84	375±130	5/5	5/5	5/5
GSH-OEt(+)	TNF-α	8	1837±765	898±562	1098±501	5/5	5/5	5/5
GSH-OEt(+)	TNF-α	12	2247±1989	1267±1217	2878±842	5/5	5/5	5/5

TNF-α (0.5μg) (obtained from BD Bioscience) was administered intraperitoneally as a mixture in 0.2 ml of pyrogen-free saline to 12-week-old Nrf2 deficient and wild type (BSO treated and untreated) mice.

D-galactosamine hydrochloride (GalN) (obtained from Nacalai Tesque) was pre-injected intravenously to increase the susceptibility of mice to hepatotoxicity 30 min before TNF-α injection. GSH-OEt (20mg/mouse) was injected intravenously 2 h before TNF-α injection to assess the importance of the intracellular GSH level. 0, 4, 8 and 12 h after injection, blood was collected and serum ALT activities were determined. Results are shown as mean ± SD for 3 to 5 mice. Survival rate indicates number of survived/all mice. (*) significantly different from wild type mice ($P < 0.05$).

Figure 1

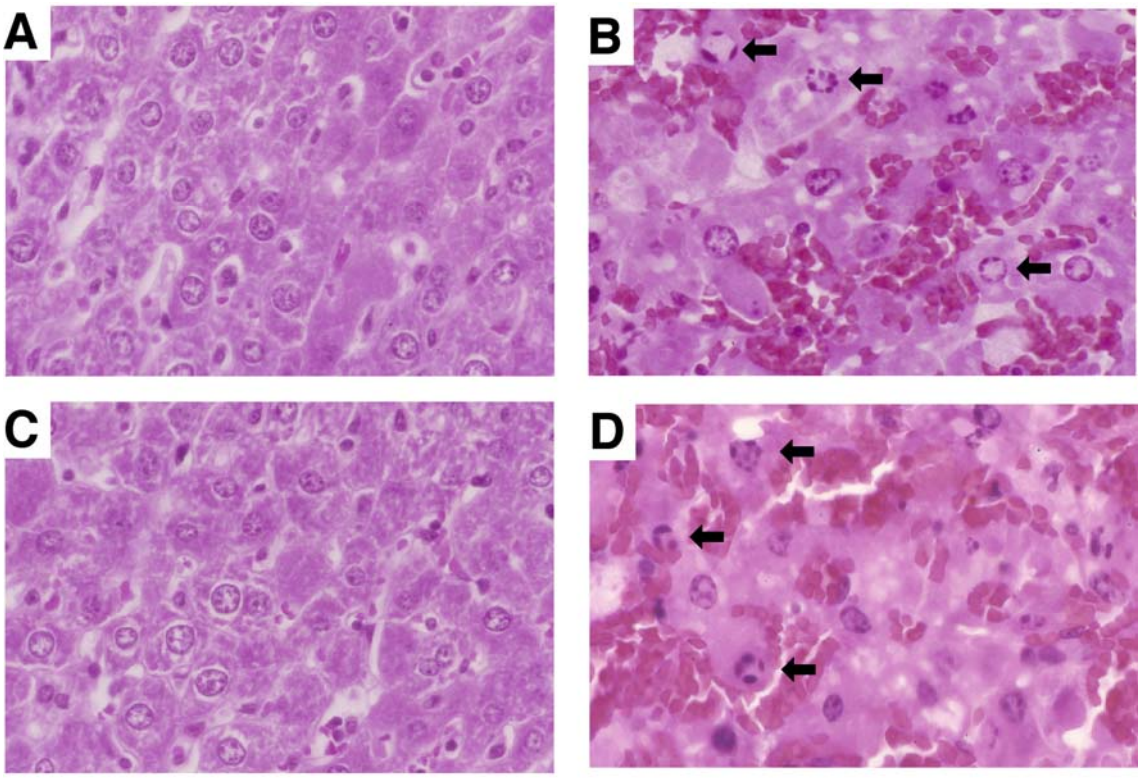

A
GSH-OEt(-)
□Nrf2 wild type (n=5)
○Nrf2 deficient (n=5)
survival rate (%)
anti-Fas antibody (ng/ml medium)
B
GSH-OEt(+) (2mM)
□Nrf2 wild type (n=5)
○Nrf2 deficient (n=5)
survival rate (%)
anti-Fas antibody (ng/ml medium)
C
GSH-OEt(-)
▲BSO untreated wild type (n=5)
△BSO treated wild type (n=5)
survival rate (%)
anti-Fas antibody (ng/ml medium)
D
GSH-OEt(+) (2mM)
▲BSO untreated wild type (n=5)
△BSO treated wild type (n=5)
survival rate (%)
anti-Fas antibody (ng/ml medium)
E
GSH (pmol/10^6 cells)
untreated
+BSO
+BSO +GSH-OEt
untreated
+GSH-OEt
wild type
Nrf2 deficient
pre-anti-Fas administration (n=4)
post-anti-Fas administration (n=4)

Effects of Nrf2 deficiency on cultured thymocytes treated with an agonistic anti-Fas antibody. The generation of Nrf2 deficient mice was previously described (Itoh *et al*., 1997). The present study utilized 12-week-old mice from the same litters with an ICR background. Freshly isolated thymocytes from Nrf2 deficient, chronically treated with BSO (6 weeks) and untreated wild type mice were incubated overnight with the indicated concentrations of anti-Fas antibody. The cells were washed with DMEM supplemented with 10% heat-inactivated fetal bovine serum, 0.5 mM 2-mercaptoethanol, 2 mM glutamine, 1 mM HEPES (pH 7.4) and antibiotics (all from Invitrogen). The cells were again washed in medium before use in subsequent tissue culture experiments. The cells (1×10^6/ml) were incubated with various concentrations of anti-Fas antibody (Jo-2, BD Bioscience) for 12 hours. Cell viability was determined by trypan blue exclusion. The cells were pre-incubated with GSH-OEt (obtained from WAKO) (2mM for 15-20 min) to increase the intracellular pool of GSH. Each Bar represents the mean ± SD. (A) Survival rate

of Nrf2 deficient thymocytes after Fas stimulation was significantly decreased ($P < 0.05$) compared with wild type thymocytes. Nrf2 deficiency increased the percentage of apoptotic thymocytes after Fas stimulation. (*) indicates significant difference from wild type thymocytes ($P < 0.05$). (B) GSH-OEt protected Nrf2 deficient thymocytes against Fas-mediated apoptosis. Thymocytes were pre-incubated with 2mM GSH-OEt. There was no significant difference in the survival rate between Nrf2 deficient and wild type thymocytes with 2mM GSH-OEt. (C) The survival of wild type thymocytes chronically treated with BSO was significantly decreased after Fas stimulation ($P < 0.05$) compared with untreated wild type thymocytes. BSO, a specific GSH synthesis inhibitory agent, increased the percentage of apoptotic thymocytes after Fas stimulation, similar to Nrf2 deficiency. (**) indicates significant difference from BSO untreated wild type thymocytes ($P < 0.05$). (D) GSH-OEt protected BSO chronically treated thymocytes against Fas-mediated apoptosis. There was no significant difference in the survival rate between BSO treated and untreated wild type thymocytes with 2mM GSH-OEt. (E) GSH levels of thymocytes were

examined pre and post anti-Fas antibody administration. GSH levels were measured using a Total Glutathione Quantification Kit (DOJINDO). The GSH levels of Nrf2 deficient thymocytes were significantly reduced compared to wild type thymocytes, being decreased to almost the same level as that of BSO treated thymocytes. GSH-OEt addition increased the GSH levels of Nrf2 deficient and BSO treated thymocytes to almost the same level as that of Nrf2 wild type thymocytes. (#) indicates significantly difference from untreated wild type thymocytes ($P < 0.05$).

Figure 2


A
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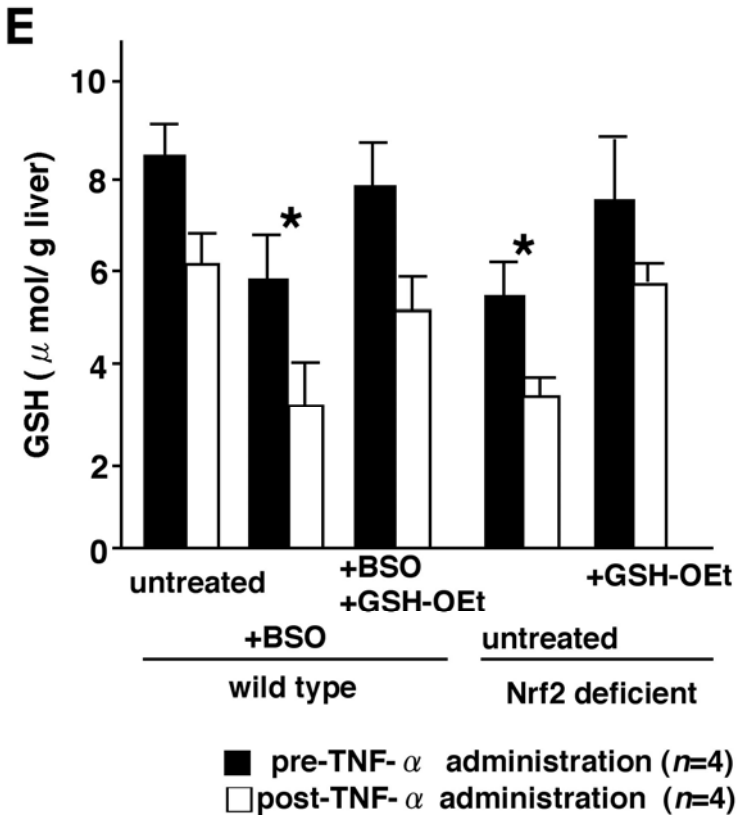
E
GSH (μ mol/ g liver)
10
8
6
4
2
0
*
*
untreated
+BSO
+GSH-OEt
+GSH-OEt
+BSO
untreated
wild type
Nrf2 deficient
pre-TNF- α administration (n=4)
post-TNF- α administration (n=4)

Nrf2 deficiency enhanced TNF-α□mediated apoptosis and GSH-OEt rescued Nrf2 deficient mice from TNF-α-mediated apoptosis. Mice were sacrificed 12 hours after TNF-α (0.5μg) injection. GalN was pre-injected intravenously 30 min before TNF-α injection. GSH-OEt (20mg) was injected 2 hours before TNF-α administration to increase intracellular glutathione levels. The liver was excised, fixed with 10% buffered formalin, sectioned at a thickness of 5□m, and stained with hematoxyline and eosin for light microscopic examination. (A) Liver section of wild type mouse: a few apoptotic hepatocytes exhibit apoptotic nuclei. (B) Nrf2 deficient mouse: numerous apoptotic hepatocytes were observed (Arrows indicate apoptotic nuclei). (C) Nrf2 deficient mouse with GSH-OEt administration: apoptotic cells were decreased by the addition of GSH-OEt. (D) BSO treated wild type mouse: numerous apoptotic hepatocytes were observed as seen in Nrf2 deficient mouse (Arrows also indicate apoptotic nuclei). (E) GSH levels of livers were examined pre and post TNF-α administration. Total GSH level was measured in homogenized liver samples. The GSH level of Nrf2 deficient livers was significantly reduced

compared to wild type being decreased to almost the same level as that of BSO treated livers. GSH-OEt addition increased the GSH levels of Nrf2 deficient and BSO treated livers to almost the same level as that of Nrf2 wild type liver. (*) indicates significantly difference from untreated wild type livers ($P < 0.05$).